

# Topolin-derived cytokinins and cytokinin oxidase inhibitors offer new tools to study in vitro plant development

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## Abstract

Exactly 20 years ago the first report about the merits of meta-topolin as an alternative of benzyl adenine in plant tissue culture was published. Since then a number of derivatives have been developed: the so called topolins. Quite a few research groups have shown that these compounds can make the difference between success or failure in the micropropagation system of a plant species. Recently another structurally related group of cytokinin oxidase inhibitors such as INCYDE and phenyl adenine have been discovered. The topolins as well as the cytokinin oxidase inhibitors are worthwhile to be added to the plant growth regulator toolbox of a tissue culture lab.

**Keywords:** topolins, Phenyl adenine, INCYDE, melia, cytokinin oxidase

## INTRODUCTION

### Topolin cytokinins

Cytokinins (CKs) play an essential role in plant micropropagation systems. Natural cytokinins are always N6-substituted purine derivatives which can be classified by the configuration of their N6-side chain as isoprenoid or aromatic cytokinins. In the early years of cytokinin research, only cytokinins with an isoprenoid side chain were thought to be endogenous compounds. Benzylaminopurine (BA) is the best-known aromatic cytokinin, which, unexpectedly was identified in mature poplar leaves, together with the riboside of orthotopolin (oTR) by Horgan et al. (1975). Strnad (1997) also isolated meta-topolin (mT) from poplar leaves. Later, many other so called topolin derivatives were found as naturally occurring cytokinins. To study the structure-activity relationship of aromatic cytokinins, (Doležal et al., 2006, 2007) synthesized systematically up to 48 aromatic cytokinins and derivatives. Their cytokinin activity was compared, with BA as standard compound, in three cytokinin bioassays: tobacco callus, wheat senescence and *Amaranthus* bioassay. The majority exhibited a high activity in all three bioassays. Concerning the substitutions on the aromatic ring, the results suggested a general trend of cytokinin activity being: meta ≥ ortho > para. As these bioassays showed strong differences in activities of the same cytokinin compounds, it may be concluded that specific cytokinins can be used to target particular plant propagation processes. Since the first report on the merits of mT (Werbrouck et al., 1996), mT and its derivatives have been employed for applied micropropagation and to solve various in vitro induced physiological disorders in many species, as reviewed by Aremu et al. (2012b).

### Cytokinin oxidase inhibitors

Cytokinin oxidases/dehydrogenases (CKX) are involved in the catabolism of endogenous cytokinins by oxidative removal of their side chain in a single enzymatic step (Schmülling et al., 2003). Hence, they play an important role in the cytokinin homeostasis. The development of a number of potent CKX inhibitors promises to offer tools to accumulate

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endogenous cytokinins in *in vitro* explants. The well-known synthetic phenyl urea cytokinins CPPU and thidiazuron are inhibitors of CKX (Chatfield and Armstrong, 1986; Laloue and Fox, 1989). Furthermore, both strongly activate the cytokinin receptors AHK3 and AHK4 (Spíchal et al., 2004; Kopečný, 2010). This dual action might explain its very strong activity in adventitious shoot formation in many dicotyledonous plant species.

Continuous efforts to synthesize purines with various substituents led to the discovery of two interesting phenyl adenine (Figure 1) derivatives (Zatloukal et al., 2008). They were called inhibitors of cytokinin degradation (INCYDE). The most powerful types were INCYDE-Cl (2-chloro-6-(3-methoxyphenyl)aminopurine) and INCYDE-F (2-fluoro-6-(3-methoxyphenyl)aminopurine) (Figure 1) and they combined a high CKX inhibition, being even stronger than TDZ, with a relatively low cytokinin receptor activation. The core molecule, phenyl adenine (PA), however, was not recognized as a CKX inhibitor at that time. Although these newly developed compounds were very promising, their limited availability restricted further development of applied micropropagation protocols. An exception was the work of Aremu et al. (2012a, 2015) in which was shown that mT and INCYDE worked synergistically in a micropropagation system of banana and produced more shoots compared with mT or INCYDE alone. Additionally, INCYDE generally favoured shoot regeneration in *Eucomis autumnalis* and promoted the accumulation of higher concentrations and varieties of endogenous cytokinins (Aremu et al., 2015).

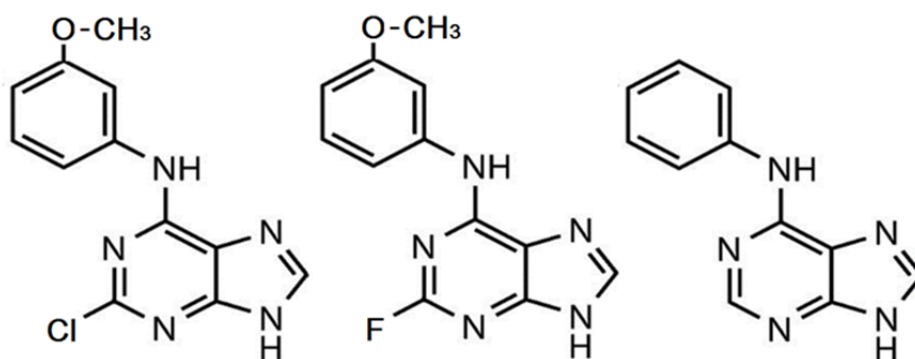


Figure 1. Phenyl adenine, INCYDE-Cl and INCYDE-F.

Some years later, PA popped up again in a chemical screening of a library of 10,000 small molecules (Motte et al., 2013). In a two-step regeneration protocol adjusted and optimized for high-throughput manipulations of root explants of *Arabidopsis thaliana* carrying the shoot regeneration marker LIGHT-DEPENDENT SHORT HYPOCOTYLS4, phenyladenine was the only compound being a potent inducer of adventitious shoots. PA activated the cytokinin receptors AHK3 and AHK4 in a bacterial receptor assay. Additionally, PA turned out to be a competitive inhibitor of CKX, leading to an accumulation of endogenous cytokinins (Motte et al., 2013).

To illustrate the effects of the new cytokinin oxidase/dehydrogenase inhibitors INCYDE-Cl and INCYDE-F and PA, the results of two experiments with leaf explants are shared. The first one with *Melia volkensii*, a hardwood multipurpose tree endemic to the semi-arid and arid areas of Kenya, the second one with tobacco, notorious for its sensitivity to cytokinins and rapid adventitious shoot formation.

## MATERIALS AND METHODS

### Adventitious shoot induction on leaf explants of *Melia volkensii*

MS medium, including macro and micro salts and vitamins (Murashige and Skoog, 1962) was supplemented with 2% sucrose and 0.7% Plant Agar. Combinations of 0, 1, 10 and 100  $\mu$ M tZ or 2iP with 0 or 10  $\mu$ M INCYDE-Cl, INCYDE-F, TDZ or CPPU were added to 10 mL medium in petri dishes ( $\varnothing$  55 mm). Every treatment consisted of 5 petri plates with 5 leaf

explants of *Melia volkensii* each, taken from in vitro stock plants. This experiment was repeated thrice and was evaluated after 50 days. The relative number of leaves with adventitious shoots was recorded. The results were not normally distributed and homoscedasticity was not fulfilled. Therefore, the Kruskal-Wallis test was chosen to examine whether the means differed statistically (95% confidence interval).

#### Adventitious shoot induction on leaf explants of *Nicotiana tabacum*

In a second experiment, the same basal medium was used. Combinations of 0, 1  $\mu$ M 2iP with 0 or 1  $\mu$ M INCYDE-Cl, PA or TDZ were added to 10 mL medium in petri dishes ( $\phi$  55 mm). Every treatment consisted of 4 petri plates with 5 leaf explants of *Nicotiana tabacum* each, taken from in vitro stock plants. This experiment was repeated thrice and was evaluated after 21 days.

## RESULTS AND DISCUSSION

#### Adventitious shoot induction on leaf explants of *Melia volkensii*

The combined results are presented in Table 1. The control treatment did not produce any shoots at all. It was not a surprise that tZ is more potent to induce adventitious shoots than 2iP. The INCYDEs not only showed a strong autonomous shoot induction capacity; they even outperformed TDZ and CPPU whether or not combined with tZ or 2iP. The effect of the INCYDES could not be improved by adding 2iP or tZ. Remarkably, the INCYDEs could awaken sleeping meristems in the secondary axils of the composed leaves (Figure 2).

Table 1. Effect of combinations of tZ or 2iP with INCYDE-Cl, INCYDE-F, TDZ or CPPU on adventitious shoot induction in *Melia volkensii* leaves.

	$\mu$ M	0	INC-Cl 10 $\mu$ M	INC-F 10 $\mu$ M	TDZ 10 $\mu$ M	CPPU 10 $\mu$ M
tZ	0	0 i	65 abc	73 a	36 efg	1 hi
	1	1 hi	73 a	70 ab	26 fgh	5 hi
	10	5 hi	73 a	70 ab	36 efg	4 hi
2iP	100	41 cdefg	69 ab	66 abc	37 defg	33 efg
	1	0 i	69 ab	62 abcd	45 bcdefg	1 hi
	10	0 i	69 ab	58 abcde	41 cdefg	4 hi
	100	16 ghi	77 a	64 abc	52 abcdef	4 hi

Means (%) followed by the same letter are not significantly different (Kruskal-Wallis).

At 10  $\mu$ M INCYDE-Cl or INCYDE-F can easily induce adventitious shoots, directly or indirectly with a callus interphase. The apparent awakening of sleeping meristems in the secondary leaf axils of composed leaves was not expected and has not been reported yet. It is not clear whether this is caused by the cytokinin properties of the INCYDEs in se, or whether they inhibited the oxidation of endogenous cytokinins already present in certain zones in the leaf explant. Detailed hormonal analysis of particular tissues and histology is needed to answer this question.

#### Adventitious shoot induction on leaf explants of *Nicotiana tabacum*

The results were visualized in Figure 3. The control medium did not induce any adventitious shoots. At a concentration of 1  $\mu$ M, PA, INC and TDZ induced numerous shoots, and combining them with 2iP also stimulated callus formation. This demonstrates that the new CKX inhibitors are as potent as TDZ. It was not possible to distinguish between cytokinin receptor activation or CKX inhibition allowing accumulation of endogenous cytokinins. Therefore, also here, detailed hormonal analysis is necessary.

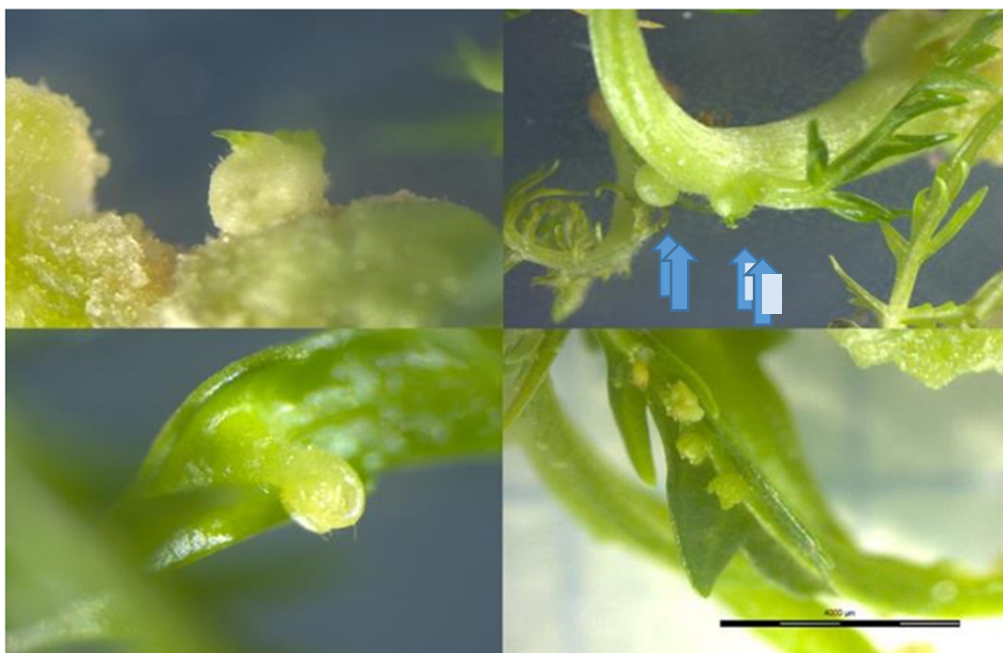


Figure 2. Adventitious shoot regeneration on leaf explants of *Melia volkensii* by 10  $\mu$ M INCYDE-Cl. The arrows point at awakening meristems in the secondary axils of the composed leaves (bar=4 mm).

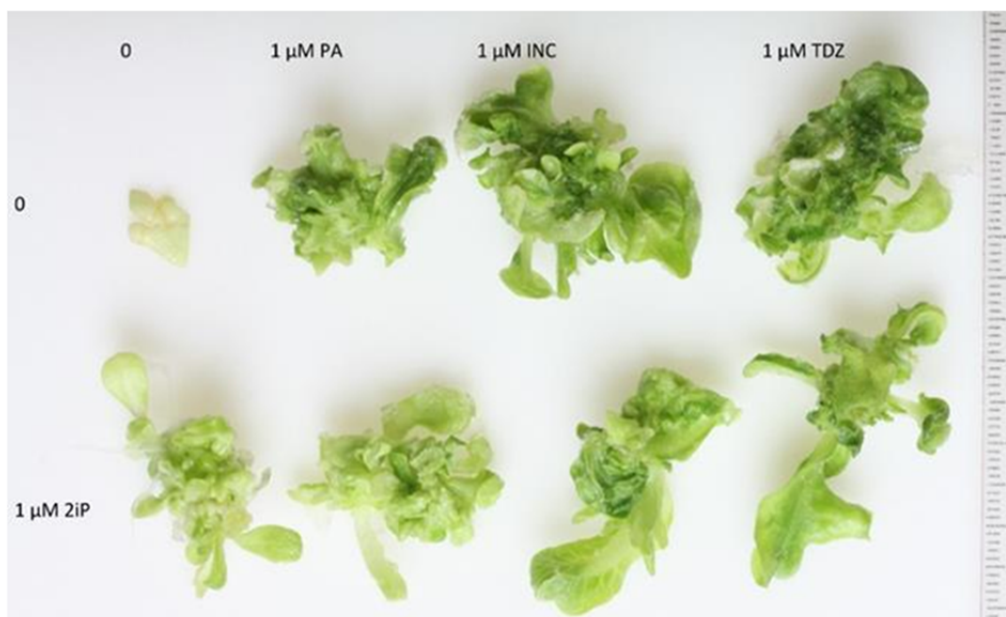


Figure 3. Adventitious shoot inducing effect of 0 or 1  $\mu$ M 2iP + 0 or 1  $\mu$ M PA, INC or TDZ in leaf explants of *Nicotiana tabacum*.

## CONCLUSION

In the past 20 years, the role of topolins has been studied in tissue cultures of many plant species. Although several research groups have shown that in many cases they are a better alternative than benzyladenine, their value still remains to be discovered by many commercial and scientific plant tissue culture laboratories. Also, PA and both INCYDEs offer

new tools to study in vitro plant development. Exploring them in various plant species might point toward new unknown organogenic pathways.

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